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14. ABSTRACT
Recently, gene rearrangements involving ETS family transcription factors have been identified in >50% of prostate cancer cases. To address roles of these ETS factors, especially ERG, the most frequently rearranged ETS gene in prostate cancer, as well as in normal prostate development, we planned to, 1. Generate conditional knockin mouse models of prostate cancer based on the newly identified TMPRSS2-ERG (or ETV1) gene arrangements; 2 Explore roles of Erg during development as well as in normal prostate by disrupting its expression in mouse; 3. Identify downstream target genes of ERG or ETV1 in human prostate cancer cell lines carrying these gene arrangements using the ChIP-on-Chip approach. During the first year of this award, we have made significant progress in establishing systems and reagents for all three aims mentioned above. Specifically, we have successfully created conditional knockin mice expressing truncated human ERG and ETV1 (as found in patients) from the endogenous mouse Tmprss2 locus. We have generated an Erg knockdown allele in mice, which would allow us to study its roles during both embryonic development and postnatal prostate development. We have also made biotinylated ERG and ETV1 in prostate cell lines, which would allow us to identify the downstream targets of these factors in prostate epithelial cells. Further studies using these animal and cell culture models would allow us to develop preclinical animal models, and to identify and validate novel therapeutic targets, for treating prostate cancer.

15. SUBJECT TERMS
Prostate cancer, gene targeting, mouse model, gene rearrangement, oncogenesis

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Introduction:

Prostate cancer is among the most frequently diagnosed cancers and the second most common cause of male cancer-related death (*1, 2*). It arises through a progression from an initial benign stage to frank malignancy, most often correlating with androgen-dependence and then androgen-independence in the late stage (*3*). Among genetic changes accompanying prostate cancer, of greatest interest are genetic "hits" in prostate epithelial cells that might represent initiating events for oncogenesis or consistent somatic mutations that specifically affect these cells. Identification of and modeling these causal genetic alterations would be invaluable in providing novel therapeutic targets and prognostic markers. Recently, chromosomal rearrangements between androgen-responsive genes, such as *TMPRSS2*, and genes encoding the ETS family transcription factors (*ERG*, *ETV1*, *ETV4*, and *ETV5*) were identified in >50% of prostate cancer cases (*4-13*). Due to their high incidence in prostate cancer, these may represent the most common gene rearrangements identified in human cancers. In addition, in analogy to the role of translocations in hematological malignancies, these chromosomal rearrangements represent either the initiating event in oncogenesis or a critical and consistent set in the cancer progression. Among ETS genes involved in these rearrangements, the most common one is *ERG*, followed by *ETV1* (*14*). Arrangements of coding regions of these ETS genes to control regions of androgen-responsive genes presumably lead to overexpression of these proto-oncogenes. In this study, we plan to create and characterize a series of mouse models based on *TMPRSS2-ERG* (or *ETV1*) gene rearrangements to study roles of these ETS genes, especially *ERG*, in development and in prostate tumorigenesis. We also plan to use biochemical approaches to identify downstream targets of ERG as well as its interaction partners. These studies would allow us to develop preclinical animal models, and to identify and validate novel therapeutic targets, for treating prostate cancer.

Body:

Specific aim 1: Establish a novel Cre/lox conditional knockin mouse model of prostate cancer based on the newly identified TMPRSS2-ERG(or ETV1) gene arrangements.

We proposed to use the Cre/lox conditional approach to model prostate cancer based on the *TMPRSS2-ERG/ETV1* chromosomal rearrangements. We planned to use two different strategies to achieve this aim. In one strategy, we proposed to generate conditional knockin alleles to express the N-terminal truncated human ERG (or ETV1, as found in patients) from the mouse endogenous *Tmprss2* locus. In a second strategy, we proposed to create knockin alleles of both *Tmprss2* and *Erg* by introducing a single loxP site to each allele, which would then permit Cre-mediated interchromosomal recombination as well as intrachromosomal excision.

For the first strategy, we knocked in the N-terminus-truncated human *ERG* or *ETV1* cDNA together with an ires-Gfp marker into the exon 2 of mouse *Tmprss2* locus. We placed a transcriptional stopper cassette together with Neo (Neo-ST) upstream of the knockin cDNA. The Neo-ST cassette is flanked by loxP sites. Cre-mediated excision would remove this cassette and activate the *Tmprss2-ERG* or *ETV1* fusion genes. Several correctly targeted independent ES clones from either *ERG* or *ETV1* knockins were injected into mouse blastocysts and a colony of germline-transmitted heterozygous knockin mice was established. In addition, we also generated several stopper-removed knockin *Tmprss2-ERG* ES cell lines *in vitro* and made chimeric male mice from them by blastocysts injection. Since in these male chimeras, *Tmprss2-ERG* has already been activated in prostate cells carrying this allele, we have started to monitor them for any prostate abnormality. By RT-PCR, we confirmed that the *Tmprss2-ERG* fusion transcript was expressed from the endogenous *Tmprss2* locus from prostate tissues of the *Tmprss2-ERG* chimeric males (Figure 1), suggesting the human *ERG* cDNA was indeed correctly targeted to the mouse *Tmprss2* locus.

For the second strategy, we aimed to introduce a single loxP site to both the *Tmprss2* and the *Erg* loci, respectively. If the two knockin loxP sites are located on the same chromosome (chromosome 16 for mice), Cre-mediated recombination would delete the ~3Mb region between them, and generate the *Tmprss2-Erg* fusion gene following deletion. If the two loxP sites are knocked into two different chromosome 16s, then Cre-medicated recombination would lead to chromosomal translocation. The *Tmprss2-Erg* fusion gene would be created following translocation. To generate such a model, we first introduced the above-described floxed Neo-ST cassette into the *Erg* locus in the intron upstream of its exon 4. This immediately gave us a knockdown allele for *Erg*, which would allow us to study the role of Erg in normal development (will discuss in the progress for specific aim 2). In ES cells carrying this *Erg* knockdown (kd) allele, we expressed Cre transiently *in vitro* to remove the

floxed Neo-ST cassette. After excision, only a single loxP site remains. We then performed sequential targeting from these ES cells to introduce the second loxP site into the *Tmprss2* locus. This was also achieved through the introduction of the floxed Neo-ST cassette, followed by the Cre-mediated removal of this cassette. Currently, we have obtained several positive ES clones from the sequential targeting. We are now in the process of characterizing these ES clones (confirming targeting, karyotyping, ascertaining whether two targeting events occurred on the same chromosome 16, or two different 16s).

To test a potential synergy between *Tmprss2-ERG* or *ETV1* fusion models and other mouse models of prostate cancer, we recently acquired *Pten* conditional knockout mice (*15*) and have started to breed this strain to our *Tmprss2-ERG* knockin mice. Both the *Pten* conditional allele and the *Tmprss2-ERG* conditional allele would be activated by breeding in the prostate-specific *PB-Cre4* transgene (*16*).

Future work would focus on characterization of the *Tmprss2-ERG* (or *ETV1*) knockin mice and determine if male mice carrying these alleles (after Cre-mediated activation) would develop hyperplasia or tumors either alone or in combination with *Pten* loss.

Specific aim 2: Explore roles of the ETS family transcription factor ERG in development and in normal prostate by making a conditional knockout allele of this gene in mouse.

As mentioned above, we generated an *Erg* knockdown allele in mice by placing a transcriptional stopper cassette into the *Erg* locus. Mice homozygous for this knockdown allele died before E11.5 during embryonic development. At E10.5, homozygous embryos appeared pale and there was only a trace amount of blood running in major vessels, suggesting a possible endothelial cell defect, or hematopoietic defect, or both (Figure 2). Since the stopper cassette we introduced is flanked by loxP site, Cre-mediated excision of this stopper cassette would allow us to restore *Erg* expression. We are currently breeding this *Erg* knockdown allele with *Tie2-Cre* (*17*), so that we can restore *Erg* expression in both endothelial and blood cells during development. This would potentially allow us to bypass the early lethality we saw in *Erg* knockdown homozygotes. If *Tie2-Cre* mediated rescue would allow homozygous mice to survive to adulthood, it would allow us to study the role of Erg in prostate development, since *Tie2-Cre* only restores *Erg* expression in endothelial and hematopoietic cells, not in prostate epithelial cells.

In addition, we are in the process of making an *Erg* conditional knockout allele by flanking its last coding exon with loxP sites. This last exon encodes the DNA binding domain of Erg which is critical for its function. For the first targeting vector we generated based on this design, we were not able to get any correctly targeted ES clones after screened several hundred clones, suggesting a genomic region very difficult to target. We then modified this original targeting vector by extending its 3' homologous arm. After screening another several hundred ES clones, recently we obtained one clone that appeared correctly targeted at the 5' arm as determined by southern blot. Currently we are working on confirming this potential clone by testing its 3' arm by southern blot.

Specific aim 3: Identify downstream target genes of ERG or ETV1 in human prostate cancer cell lines carrying these gene arrangements using the ChIP-on-Chip approach.

To identify new ERG and ETV1 target genes and collaborating factors in prostate development and disease, we used LNCaP and VCaP prostate cancer cell lines. LNCaP cells overexpress ETV1 under the *HNRPA2B1* prostate-specific promoter (*12*), whereas VCaP cells are characterized by the presence of the TMPRSS2-ERG fusion protein (*9*). Both prostate cancer cell lines displayed high ERG (VCaP) and ETV1 (LNCaP) levels that increased after androgen stimulation.

Expressing profiling by microarrays has become a standard method to study cancer. To analyze further the role of ERG and ETV1 in the prostate, we have performed siRNA-silencing experiments. Eight and 48 hours after transient transfection with ERG and ETV1 specific siRNA from Dharmacon, LNCaP and VCaP cells were harvested. We hybridized total RNA prepared from androgen stimulated ERG and ETV1 siRNA silenced experiments onto Affymetrix human whole genome expression array U133Plus2.0 in the DFCI Gene Expression Core. From the recently obtained array data, we may see a signature of self-renewal genes or signaling pathway genes, which are crucial in prostate cancer biology. We are also interested in cell migration and invasion genes, since they play a pivotal role in cancer progression.

Moreover, to identify potential target genes of ERG and ETV1 in prostate cancer, we planned to perform genome wide location analysis for mapping the *in vivo* ERG and ETV1 binding (or target) sites in normal and cancer cell

lines. A key requirement for the success of this analysis is a reliable antibody that recognizes the protein of interest specifically in an immunoprecipitation assay. We tested several commercially available antibodies against human ERG and ETV1, and unfortunately they are not good for the process. Thus, to identify ERG and ETV1 binding sites, we utilized a metabolic biotin labeling approach (*18, 19*), in which the *ERG* and *ETV1* cDNA were engineered to contain an amino-terminal Flag epitope and a short peptide tag that serves as a substrate for *in vivo* biotinylation. This cDNA was then expressed in LNCaP, and VCaP cells (which were isolated from metastasic prostate cancer) previously engineered to ectopically express the *Escherichia coli* biotin ligase *BirA*. The exceptionally strong interaction between biotin and streptavidin allows for the efficient recovery of biotinylated protein with cross-linked DNA, as in conventional chromatin immunoprecipitation (ChIP) experiments. In this period, we generated LNCaP and VCaP cells that contain the *bioERG* and *bioETV1*, joined to biotin ligase BirA by retroviral infection. In the near future, material recovered in this "bio-ChIP" method will be hybridized to Affymetrix promoter arrays to identify putative target genes of ERG or ETV1. This approach (bio-ChIP-on-Chip) has been successfully utilized in our lab to identify target genes of pluripotency genes in embryonic stem cells (*18*). By coupling ChIP-on-Chip information with the transcriptome analysis, we will identify candidate target genes of ERG and ETV1, which will be then validated by RT-PCR and ChIP assays. In addition, data comparing prostate normal and cancer cells will allow us to identify genetic/epigenetic changes involved in cancer initiation and progression.

Key research accomplishments:

- Successfully generated *Tmprss2-ERG* or *ETV1* knockin mice expressing truncated human ERG or ETV1 from the endogenous mouse *Tmprss2* locus.
- Generated a rescuable *Erg* knockdown allele in mice that would allow us to study roles of Erg during embryonic development and in prostate.
- Generated Flag-tagged biotinylated ERG or ETV1 (*bioERG* and *bioETV1*) for biochemical studies.

Reportable outcomes:

None

Conclusion:

We planned to use both a series of mouse models and biochemical approaches to study the mechanisms of pathogenesis in human prostate cancer with *TMPRSS2-ERG* (or *ETV1*) gene fusions. During the first year of this award, we have made significant progress, including successfully generating the conditional knockin mice carrying the *Tmprss2-ERG* (or *ETV1*) fusion allele, generation and initial characterization of an *Erg* knockdown allele, and establishment of a biotinylation system for both ERG and ETV1 in human prostate cancer cell lines for biochemical studies. Further studies using these animal and cell culture models would allow us to develop preclinical animal models, as well as to identify and validate novel therapeutic targets, for treating prostate cancer.

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Appendices:

None

Supporting Data:

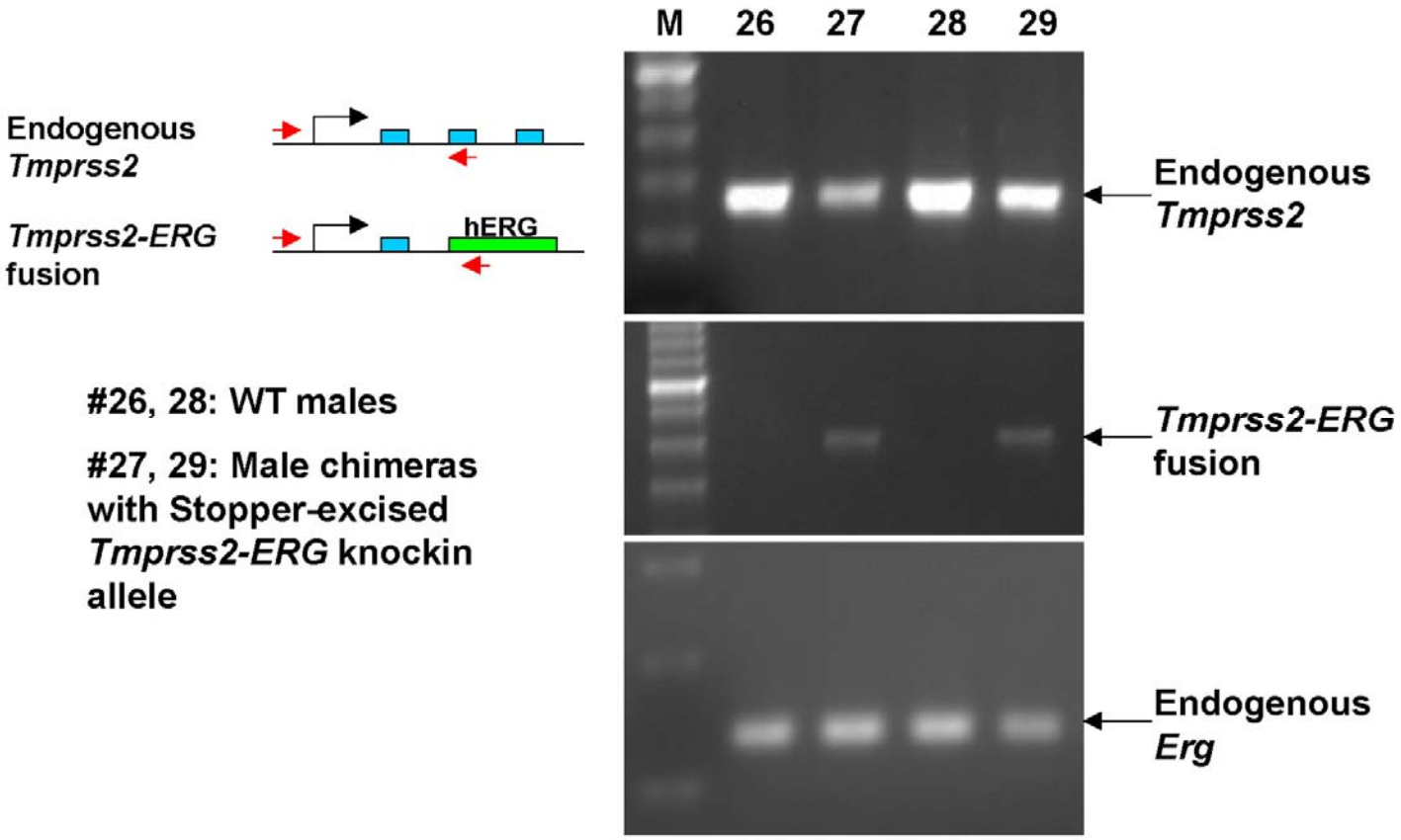

Figure 1. Expression of the *Tmprss2-ERG* fusion transcript from the Stopper-excised *Tmprss2-ERG* knockin allele (thus activated) determined by RT-PCR. PCR primers used to detect the endogenous *Tmprss2* transcript and the *Tmprss2-ERG* fusion transcript are shown in the schematic drawing (red arrows). PCR primers used to detect the endogenous mouse *Erg* are located in exons further downstream (not shown).

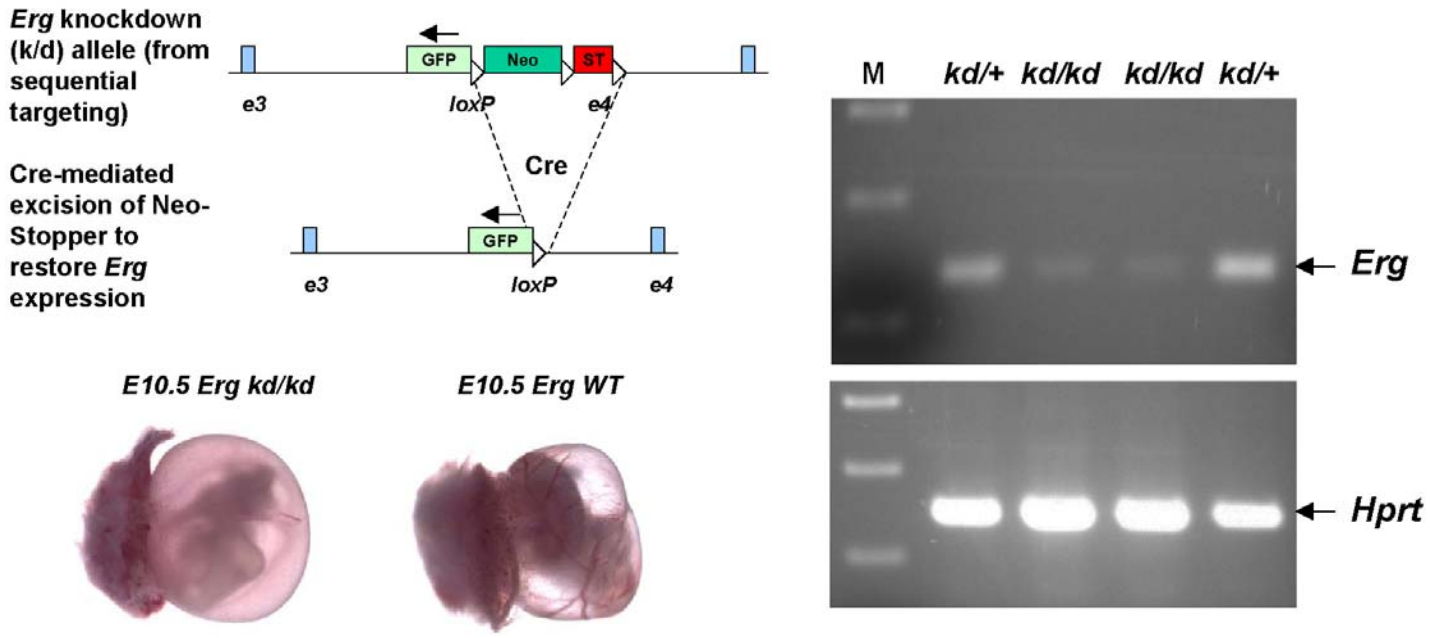

Figure 2. *Erg* knockdown allele. Mouse embryos homozygous for this *Erg* knockdown (kd) allele die before E11.5, possibly due to a hematopoietic defect, or vascular defect, or both. Dramatic reduction of *Erg* expression was detected by RT-PCR in hematopoietic cells derived from E9.5 yolk sac progenitors. *Erg* expression can be restored by Cre-mediated excision of the Neo-Stopper (ST) cassette.